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Improvements on environmental DNA extraction and purification procedures for matagenomic analysis

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Abstract: Our previously described environmental DNA extraction method has been widely used in environmental microbial community analysis. However, residual humic substances may remain with obtained environmental DNA, which interferes downstream molecular analyses. To remedy this situation, two DNA extraction buffers (PIPES and Tris-HCl) and four purification strategies including our new modified low melting point gel purification method and three commercial kits from QIAEX, Omega and Promega were evaluated with diverse soil samples. The PIPES buffer (pH 6.5) is found to be more effective for removing the humic substances, but it leads to lower DNA yield and causes more severe DNA shearing than using the Tris-HCl buffer (pH 8.0). Gel purification and the Promega purification kit achieve much higher DNA recoveries than QIAEX or Omega kit, and higher purity of DNA is obtained by gel purification than by the Promega kit with both DNA extraction buffers mentioned above. Considering all results together, two alternative methods for DNA extraction and purification are proposed: one uses Tris-HCl buffer extraction and gel purification as the primary approach when the amount of soil or biomass is not a major concern, and the other uses PIPES buffer extraction and the Promega kit purification when severe DNA shearing and/or limited biomass occurs. Purified DNA samples by both methods are amenable for use as templates for whole community genome amplifications and PCR amplifications of bacterial 16S rRNA genes. It is demonstrated that these two alternative methods could be applied to a wide variety of environmental samples.

Key words: DNA extraction; DNA purification; metagenomic analysis; GeoChip; pyrosequencing

1 Introduction

High-throughput metagenomic methods, such as Phylochip, GeoChip, pyrosequencing, and metagenome sequencing [1−4] have greatly advanced environmental microbiology. The success of these techniques for microbial community analysis largely relies on the quality of environmental DNA obtained [5]. A number of methods have been developed for DNA extraction and purification from different kinds of environmental samples in recent years. They can be categorized as either indirect [6] or direct [7] methods. Indirect extraction means that cells are first isolated from soil and then DNA is extracted from cell pellet. Direct extraction means that cells lysis occurs within the soil or sediment matrix, and then DNA is extracted. Direct methods have been more widely adopted due to easier manipulation, less time consuming, higher DNA recovery, and smaller amount of sample, and efficient cell lysis has been the focus for most of these methods, in which enzymatic, chemical, and physical treatments or their combinations have been used [7]. Previously, we described a method [8] combining grinding, freeze-thawing and SDS to achieve high DNA recovery from environmental samples without severe DNA shearing, and this method has been

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widely adopted for DNA extraction from soil, marine sediments and other habitats [9−10].

Humic substances, phenolic compounds, polysaccharides and heavy metals are widespread in soil and often co-precipitate with DNA during extraction [11]. These compounds may cause degradation of nucleic acids, inhibition of enzymatic reactions, reduction in primer specificity, and thus experimental biases and failures [11]. To remove these contaminants, a variety of methods have been developed, including gel filtration using Sepharose and Sephadex [12], pretreatment of soil using aluminum sulfate [13], addition of hexadecyltrimethylammonium bromide (CTAB) [14], or polyvinylpolypyrrolidone (PVPP) [8, 15] in extraction buffers, cesium chloride density gradient ultracentrifugation [16], captured by magnetic beads [17], chromatographic separation [8, 18], and low melting point (LMP) agarose gel electrophoresis [19]. However, the effectiveness of these different methods varies with different soil samples [20]. LMP agarose gel electrophoresis has been used in two ways: 1) electrophoresis followed by gel or column purification [8, 19], which is limited by low DNA recovery; 2) embedded (DNA or cells) gel electrophoresis [21], which can only handle small amount of DNA or cells. However, both of these methods were ineffective to remove the residual agarose.

The objective of this work was to improve our previously described DNA extraction method [8] by developing efficient DNA purification methods for a broad range of environmental samples. Two modifications were evaluated in this work: 1) Use of a low pH PIPES buffer (pH 6.5) as an alternative DNA extraction buffer to limit the amount of co-extracted or co-precipitated humic substances with DNA during DNA extraction; 2) A LMP gel electrophoresis and phenol extraction method (gel purification, described below) was adapted [22] for a large-scale DNA purification. Our results suggest two alternative procedures for DNA extraction and purification: 1) DNA extraction using Tris-HCl buffer followed by gel purification; 2) DNA extraction with low pH PIPES buffer followed by purification using a Promega kit for samples with low microbial biomass or severe DNA shearing caused by sampling, transport or storage. This work provides two effective environmental DNA extraction approaches and firstly applies the low melting gel purification methods to environmental samples, which will accelerate the development of metagenomic study.

2 Materials and methods

2.1 Site information and soil collection

A total of 136 soil samples were selected from current projects in our lab, which were focused on the analysis of diversity, composition, and structure of soil microbial communities (Table 1). Twenty-four samples were taken from a tallgrass prairie ecosystem (warming site) with warming experiments conducted near Norman, OK, USA. Five samples were from an arsenic phytoremediation experimental station (AC) at Dengjiatang Village, Chenzhou, central south China. Thirty-seven and fourteen samples were from the University of Illinois Urbana Champaign's soybean free

Table 1 Information of samples used in this work

Sample site	Number of Samples	Coordinates	Elevation/m	Location
Warming site	24	34°58′54″N; 97°31′14″W	324	Kessler Farm Field Laboratory, Great Plain Apiaries, OK, USA
AC	5	25°48′N; 113°02′E	185	Arsenic phytoremediation experimental station, Hunan, China
Maize FACE	14	40°03′21.3″N; 88°12′3.4″W	228	SoyFACE research site, Illinois, USA
SoyFACE	37	40°03′21.3″N; 88°12′3.4″W	228	SoyFACE research site, Illinois, USA
SJYNR	7	31°39′−36°16′N, 89°24′−102°23′E	3 400−4 813	San Jiang Yuan Nature Reserve, Xizang, China
CCESR	15	45°24′31″N; 93°12′3″W	—	Cedar Creek Natural History Area, Minnesota, USA
PHACE	10	41°11′N; 104°54′W	1 930	USDA-ARS High Plains Grasslands Research Station, WY, USA
ORNL FACE	24	35°54′N; 84°20′W	230	Oak Ridge National Environmental Research Park, TN, USA

air concentration enrichment (SoyFACE and MaizeFACE, respectively). Seven samples were from the San Jiang Yuan Nature Reserve (SJYNR) in Xizang Autonomous region, China. Fifteen samples were collected from the Cedar Creek Ecosystem Science Reserve (CCESR) from a long-term experimental field (CO_2 enrichment) at Cedar Creek Natural History Area, MN, USA. Ten soil samples were from the Prairie heating and CO_2 enrichment (PHACE) experiment site at the USDA-ARS High Plains Grasslands Research Station, near Cheyenne, WY, USA. Twenty-four soil samples were taken from the Oak Ridge free-air CO_2 enrichment (FACE) site located at the Oak Ridge National Environmental Research Park (ORNL) in TN, USA. Approximately 20 g of surface soil (0−15 cm) was taken from each field plot, and all soils were kept on dry ice or stored at 4 °C during transportation and −20 °C in laboratory.

2.2 Modified DNA extraction protocol

The DNA extraction procedure described here was modified from a previous method [8]. 5 g of soil and 2 g of sterile sand were mixed in a sterile mortar with liquid nitrogen, and the sample was ground after the liquid nitrogen had evaporated. This was repeated twice more. The frozen sample was transferred to a 50 mL polypropylene centrifuge tube and 16.5 mL extraction buffer [0.1 mol/L EDTA, 1.5 mol/L NaCl, 1% CTAB, and 0.1 mol/L PIPES sodium salt (for PIPES buffer, pH=6.5) or 0.1 mol/L Tris-HCl and 0.1 mol/L NaH_2PO_4 and Na_2HPO_4 (for Tris-HCl buffer, pH=8.0)] was added, followed by 61 μL proteinase K (10 mg/mL). The sample was incubated for 30 min at 37 °C with gentle inversion every 5−10 min, after which 1.83 mL of 20% SDS was added, and incubated at 65 °C for 2 h with gentle inversion every 15−30 min. Following the incubation, the sample was centrifuged for 20 min (3 600*g*) at room temperature (RT). The supernatant was transferred to a clean 50 mL polypropylene tube, and 6 mL of extraction buffer and 0.67 mL of 20% SDS were added to the remaining soil and sand pellet, mixed, incubated at 65 °C for 15 min, and centrifuged again for 10 min (3 600*g*, RT). The combined supernatants were then extracted with an equal volume of chloroform: isoamyl alcohol [24:1 (volume ratio)] for 5−10 min by continuous inversion. The sample was centrifuged (3 600*g*, 20 min, RT) and the aqueous phase was transferred to a clean 50 mL tube and extracted again. The aqueous phase was then transferred to a translucent Oak Ridge tube, precipitated with 0.6 times volumes of 2-isopropanol and incubated at −20 °C overnight. The samples were incubated in a 37 °C water bath for 15−30 min and then centrifuged (10 000*g*, 30 min, RT) to precipitate the DNA. The supernatant was transferred to a clean tube and stored until the presence of DNA in the pellet was verified. The pellet was washed with 1 mL of ice-cold 70 % ethanol and air dried for 10−15 min. The dried pellet was dissolved in 50−500 μL of pre-warmed (50 °C) nuclease-free water.

2.3 DNA purification methods

DNA was purified using either one of three commercial DNA purification kits, QIAEX II gel extraction (QIAEX, Valencia, CA), E.Z.N.A.® gel purification (Omega, Frederick, Colorado), and Wizard® Plus Midipreps DNA Purification System (Promega, Madison, WI), or gel purification. For commercial DNA purification kits, all procedures were carried out following manufactures' protocols except that modifications were made with Wizard® Plus Midipreps DNA Purification System by incubating the column at 80 °C for 10 min three times so that more DNA was eluted. Triplicates of 10.0 μg of crude DNA were used for three purification methods, respectively.

The gel purification procedure was modified from a previously described method [22]. A 0.5% LMP agarose gel was prepared with 1 X TAE and an appropriate amount of ethidium bromide. Large toothed gel combs were used to obtain wells that could hold 80−120 μL of sample. Gels were allowed to solidify for at least 2 h before use. 10.0 μg crude DNA extract was mixed with DNA gel loading buffer (0.02% bromophenol blue, 0.02% xylene cyanol FF, 0.02 mol/L EDTA, pH 8.0 and 5% glycerol in molecular biology grade water) and subjected to electrophoresis. The gel ran slowly (e.g. 5 V/cm for 6−8 h or 3 V/cm for 14 h) to obtain good DNA separation from contaminants and to prevent gel deformation. Gel slices containing large relative molecular mass (HMW) DNA were excised and placed in a 15 mL capped polypropylene tube, melted at 65 °C water-bath for 5−10 min with gentle inversion every 2−3 min during incubation. An equal volume of 65 °C nuclease free water was added, mixed well and then cooled at RT for 2 min. After cooling, an equal volume of cold, buffer-saturated phenol (pH 7.5−8.0) was added and mixed by continuous inversion for 5−10 min. The phenol extracted mixture was placed on ice for 5 min and then centrifuged (6 000*g*, 5 min, RT). The resulting aqueous layer was transferred to a fresh tube and placed on ice. Fresh TE buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA] and phenol (0.8:1, TE: phenol) were added to extract organic phase for the second time. An equal volume of chloroform: isoamyl alcohol [24:1 (volume ratio)] was added to the combined aqueous layers and mixed by inversion for 5 min. The sample was again centrifuged (6 000*g*, 5 min, RT) and the aqueous layer was transferred to another clean tube. Chloroform: isoamyl alcohol extraction was repeated twice and the

aqueous layers were combined. An equal volume of 2-butanol was then added to the recovered aqueous layer, mixed by inversion for 5 min, and centrifuged (6 000*g*, 5 min, RT). The upper 2-butanol layer was removed and discarded. Butanol extraction was repeated at least 3−4 times until the aqueous volume had been reduced to ~400 μL. The aqueous layer was supplemented with 1:10 (volume ratio) of 3 mol/L NaOAc (pH 5.2) and 2-times volume of ice-cold 100% ethanol to precipitate the purified DNA at −20 °C for 2 h or overnight. The sample was then incubated at 37 °C for 15−30 min and centrifuged (10 000*g*, 30 min, RT) to precipitate the DNA. The pellet was washed with 70% ethanol and air dried prior to re-suspension in a small volume (50−100 μL) of nuclease-free water.

2.4 Measurements of DNA quality and quantity

Generally, three methods were used to measure the quality and quantity of extracted or/and purified DNA samples. 1) Gel electrophoresis was proceeded with 1% agarose gel stained with 0.5 μg/mL ethidium bromide, 2) ratios of $A_{260/280}$ and $A_{260/230}$ absorbance were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and 3) double stranded DNA(dsDNA) was quantified by PicoGreen with a FLUOstar Optima (BMG Labtech, Jena, Germany). Meanwhile, the concentration of humic substances was determined at A_{340} with a UV-Vis spectrometer (Hewlett Packard) using pure humic acids as the standards.

2.5 DNA amplification protocol

PCR amplifications of bacterial 16S rRNA genes and whole community genome amplification (WCGA) [23] were used to further evaluate the quality of obtained DNA samples. First, PCR amplifications were conducted in reaction mixtures containing 100 ng of DNA template, 1×PCR buffer [10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl and 2 mmol/L $MgCl_2$], 2 mmol/L dNTPs, 5 pmol/L each of the forward and reverse primers, 2.5 U Taq (Invitrogen), and water to a final volume of 50 μL. PCR was performed with the primer set Bac27F (5'-AGA GTT TGA TCM TGG CTC AG -3') and 1492R (5′- CGG TTA CCT TGT TAC GAC TT-3′). The thermal cycling protocol was used, including an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s. A final extension step of 72 °C for 7 min was also used. Negative controls were performed for all PCR reactions. Second, whole community genome amplifications were carried out with 100 ng of DNA using the Templiphi kit (GE Healthcare, Piscataway, NJ, USA), and the addition of 0.1 μmol/L spermidine and 260 ng/μL single stranded binding protein was used to improve the amplification efficiency and reduce biases [23].

3 Results

3.1 Evaluation of PIPES and Tris-HCl extraction buffers

Soil (4.0 g) sample from one ORNL FACE site (see Table 1) was used for evaluation of PIPES and Tris-HCl buffers. The color of sample supernatants extracted with PIPES buffer is much lighter than that with the Tris-HCl buffer, suggesting that DNA samples extracted with the PIPES buffer contain lower concentrations of humic substances. This is confirmed by the absorbance values of the crude DNA solutions at 340 nm, which are (0.235 7±0.000 3) for the PIPES buffer, and (0.643 7± 0.003 8) for the Tris-HCl buffer, respectively.

Crude DNA was then extracted in triplicate from 1, 2, 3, 4, or 5 g soil of a single soil sample (2UC) randomly selected from the University of Oklahoma (OU) warming site (see Table 1) using Tris-HCl or PIPES buffer. The $A_{260/230}$ and $A_{260/280}$ ratios of the crude DNA are much higher with PIPES than with Tris-HCl. For example, the DNA from 5 g of soil extracted by PIPES has $A_{260/280}$ and $A_{260/230}$ ratios of (1.787±0.007) and (1.290±0.023), respectively, while those extracted by the Tris-HCl buffer are (1.363±0.030) and (0.980±0.032), respectively (see Table 2). In addition, the $A_{260/280}$ ratios of the crude DNA extracted by the Tris-HCl buffer correlate well with soil amount (r^2=0.793 and P=0.04), but no significant correlation is observed when the PIPES buffer is used (see Fig. 1). These results indicate that the PIPES buffer improves the crude DNA

Table 2 Yield and quality of crude DNA extracted by PIPES or Tris-HCl buffer

Mass of soil/g	DNA extracted by Tris-HCl buffer			DNA extracted by PIPES buffer		
	$A_{260/280}$	$A_{260/230}$	DNA amount*	$A_{260/280}$	$A_{260/230}$	DNA amount*
1	1.457±0.009	0.993±0.013	12.67±1.06	1.757±0.009	1.000±0.087	4.65±0.54
2	1.440±0.001	0.900±0.001	26.37±1.62	1.773±0.024	1.260±0.012	11.51±1.29
3	1.430±0.012	0.860±0.029	26.16±5.76	1.723±0.009	1.113±0.062	14.45±1.31
4	1.427±0.009	0.903±0.009	38.82±2.11	1.710±0.010	1.163±0.048	17.52±3.16
5	1.363±0.030	0.980±0.032	45.64±2.98	1.787±0.007	1.290±0.023	29.61±1.88

*Total crude DNA amount extracted from different amounts of soil (dry weight).

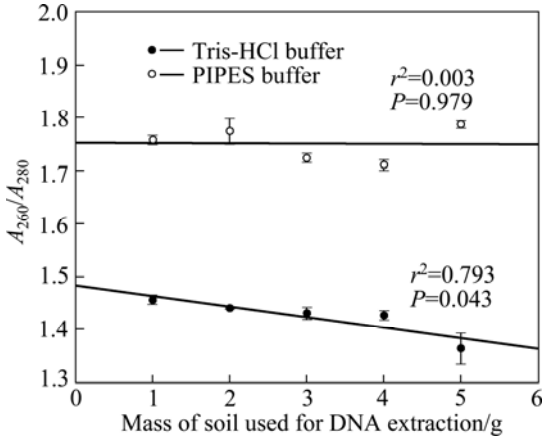

Fig. 1 Linear correlations between soil mass and $A_{260/280}$ ratio of DNA extracted by Tris-HCl or PIPES buffer from warming sample (2UC) taken from OU warming site

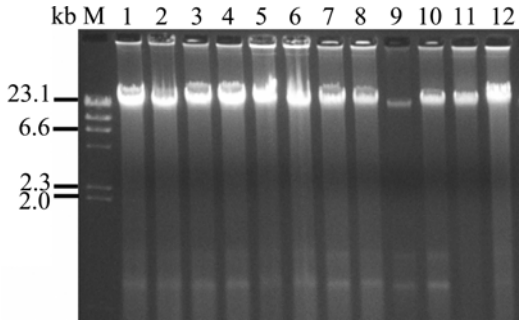

Fig. 2 Gel electrophoresis image of raw DNA extracted by Tris-HCl or PIPES buffer from six OU warming site soils (Tris-HCl buffer, lanes 1−6; PIPES buffer, lanes 7−12; Samples 1 and 7, 2 and 8, 3 and 9, 4 and 10, 5 and 11, 6 and 12 from same soil samples, respectively)

quality, possibly by preventing the extraction of some humic substances.

All crude DNA samples have large relative molecular mass (≥23 kb) (see Fig. 2), suggesting that neither PIPES buffer or Tris-HCl buffer causes severe shearing of the DNA. Total crude DNA amount is positively correlated with the soil amount with both Tris-HCl (r^2=0.94 and P=0.004) and PIPES (r^2=0.96 and P=0.002) buffers. Nevertheless, all extractions performed with the Tris-HCl buffer produce higher yields of crude DNA than those with the PIPES buffer (see Table 2). For example, the DNA yield of 5 g soil is 45.64 μg with the Tris-HCl buffer and 29.61 μg with the PIPES buffer, respectively (see Table 2). The results indicate that extraction with the PIPES buffer can obtain relatively high quality crude DNA with low yield, while extraction with the Tris-HCl buffer can obtain relatively low quality crude DNA with high yield.

3.2 Evaluation of DNA purification methods

To evaluate four DNA purification methods, approximately 10 μg of crude DNA extracted from 5 g of soil from sample 2UC with either Tris-HCl or PIPES buffer was purified by using the Promega, QIAEX II, or Omega kit or the gel purification method. The Promega kit results in a higher recovery for Tris-HCl-extracted DNA ((82.23±1.72)%) than PIPES-extracted DNA ((76.0±4.51)%), but the $A_{260/280}$ and $A_{260/230}$ ratios of purified DNA are generally higher (1.72±0.03, 1.91±0.01) for PIPES-extracted DNA than Tris-HCl-extracted DNA (1.66±0.01, 1.45±0.02) (see Table 3). In comparison, gel purification generally results in high purity DNA for both buffers (with $A_{260/280}$=1.73±0.01 and $A_{260/230}$=1.85±0.03 for Tris-HCl-extracted DNA, and $A_{260/280}$=1.77±0.02 and $A_{260/230}$=2.22±0.02 for PIPES-extracted DNA). The recovery is much higher for Tris-HCl-extracted DNA ((73.27±1.2)%) than for PIPES-extracted DNA ((26.34±3.46)%) (see Table 3). In a sharp contrast to the Promega kit and gel purification, poor recoveries are observed with the Qiagen ((13.31±1.75)% for the Tris-HCl buffer, and (8.09±0.26)% for the PIPES buffer), or Omega ((10.93±1.82)% for the Tris-HCl buffer, and (7.03±0.73)% for the PIPES buffer) kit (see Table 3). These results, taken together with the buffer comparisons, indicate that the combination of DNA extraction and purification strategies can obtain high purity DNA of environmental samples with high recovery: 1) Tris-HCl buffer extraction and gel purification, and 2) PIPES buffer extraction and purification with the Promega kit.

3.3 Evaluation of two improved DNA extraction and purification procedures

To further evaluate two approaches of DNA extraction−purification, whole community genome amplification (WCGA) and PCR amplification of

Table 3 Recovery and quality of purified DNA

Method	DNA extracted by Tris-HCl buffer			DNA extracted by PIPES buffer		
	$A_{260/280}$	$A_{260/230}$	Recovery [a]/%	$A_{260/280}$	$A_{260/230}$	Recovery [a]/%
Promega	1.66±0.01	1.45±0.02	82.23±1.72[b]	1.72±0.03	1.91±0.01	76.00±4.51[b]
Gel purification	1.73±0.01	1.85±0.03	73.27±1.20	1.77±0.02	2.22±0.02	26.34±3.46
Omega	1.86±0.03	1.73±0.03	13.31±1.75	1.75±0.05	0.30±0.07	8.09±0.26
Qigex II	2.00±0.09	1.39±0.06	10.93±1.82	1.64±0.01	1.05±0.16	7.03±0.73

a−Mean±SE (n=3); b−Yield decreases to 30% if modified elution step is not followed.

bacterial 16S rRNA genes were performed with six purified DNA samples (2UC, 2UW, 3UC, 3UW, 5UC and 5UW) taken from the OU warming site. First, consistent with the above results, high purity DNA ($A_{260/280}$ ratios of ~1.8 and $A_{260/230}$ ratios of ~1.7) is obtained from all six DNA samples extracted with the Tris-HCl buffer and purified by gel electrophoresis, while DNA samples extracted with the PIPES buffer and purified by the Promega kit have more variable $A_{260/230}$ ratios (data not shown). Second, the whole community genome amplification is successful for all DNA obtained by Tris-HCl extraction and gel purification, while it is successful for only some samples extracted with PIPES and purified by the Promega kit. Also, the amplification is not successful when crude DNA samples are directly used (see Fig. 3). In addition, when all DNA samples are used to perform PCR amplification of bacterial 16S rRNA genes, similar results are observed (see Fig. 4). These results suggest that the method with Tris-HCl extraction and gel purification is robust and reliable, and that the method with PIPES extraction and Promega kit purification may be chosen as an alternative when biomass is limited.

3.4 Application of Tris-HCl extraction and gel purification to environmental samples

To evaluate the universality of the Tris-HCl and gel purification procedure, we prepared DNA for GeoChip analysis from 136 soil samples from eight experimental sites (see Table 1). The purified DNA yields vary, ranging from 4.44 to 28.75 μg per 5 g soil. The $A_{260/280}$ (1.66−1.80) and $A_{260/230}$ (≥1.81) ratios of all purified DNA samples are qualified for most microbial community molecular analyses (see Table 4). All DNA extracts are successfully used for the whole community genome amplification, cyanine dye labeling, and GeoChip hybridization (data not shown). These results suggest that the combination of Tris-HCl and gel purification is applicable to a wide variety of environmental samples.

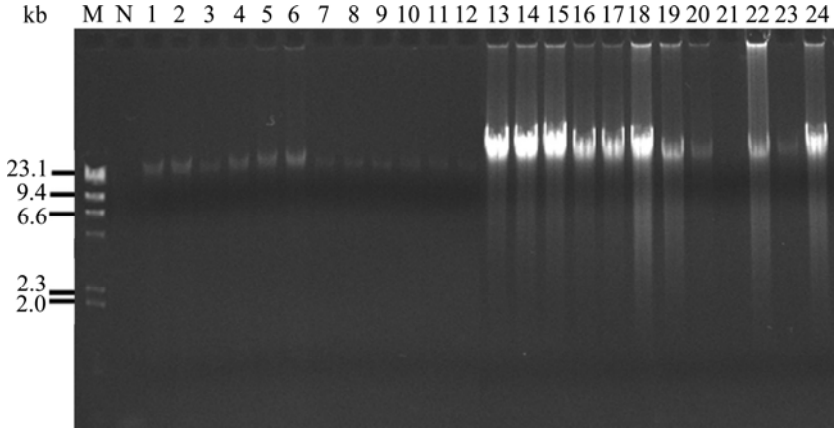

Fig. 3 Gel electrophoresis image for whole community genome amplified DNA from crude and purified DNA of OU warming site soil samples (2UC, 2UW, 3UC, 3UW, 5UC and 5UW): Lane M: HindIII-cut bacteriophage lambda molecular size marker (200 ng); Lane N: Negative control for amplification; Lanes 1−6: Raw DNA extracted by Tris-HCl buffer; Lanes 7−12: Raw DNA extracted by PIPES buffer; Lanes 13−18: DNA extracted by Tris-HCl buffer and purified by gel purification; Lanes 19−24: DNA extracted by PIPES buffer and purified by Promega kit

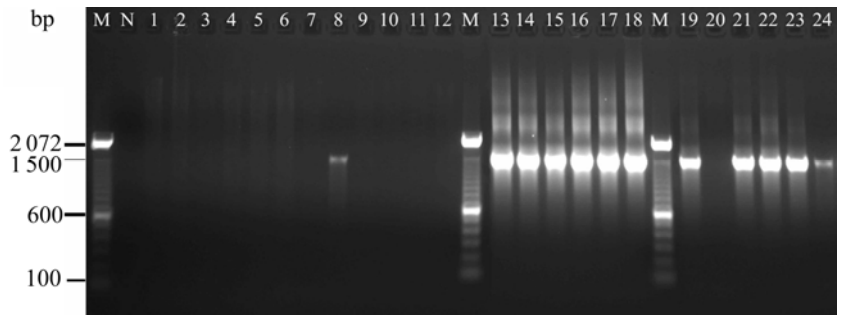

Fig. 4 Gel electrophoresis image for bacterial 16S rRNA PCR-amplified DNA from crude and purified DNA of OU warming site soil samples (2UC, 2UW, 3UC, 3UW, 5UC, and 5UW): Lane M: 100 bp molecular size marker (200 ng); Lane N: Negative control for amplification; Lanes 1−6: Raw DNA extracted by Tris-HCl buffer; Lanes 7−12: Raw DNA extracted by PIPES buffer; Lanes 13−18: DNA extracted by Tris-HCl buffer and purified by gel purification; Lanes 19−24: DNA extracted by PIPES buffer and purified by Promega kit

Table 4 Yield and purity of DNA extracted by Tris-HCl buffer (pH 8.0) and purified by gel electrophoresis from soils of varied sources

Sample site	Number of samples	$A_{260/280}$	$A_{260/230}$	Total recovered DNA[a]/μg
Warming site	24	1.762±0.010	1.834±0.033	10.716±1.122
AC	5	1.702±0.010	1.996±0.049	17.160±3.591
Maize face	14	1.776±0.012	1.891±0.018	5.804±0.609
Soyface	37	1.658±0.010	1.839±0.020	25.638±2.198
SJYNR	7	1.801±0.003	2.067±0.008	4.436±0.330
CCESR	15	1.699±0.012	1.870±0.027	5.497±0.653
PHACE	10	1.749±0.013	2.065±0.021	28.746±2.341
ORNL	24	1.722±0.009	1.814±0.014	8.718±0.326

[a] DNA yield after purification.

4 Discussion

Our published DNA extraction method, which combines grinding, freeze-thawing, and SDS lysis [8], has been widely used to recover high yields of HMW DNA from a range of environmental samples. However, this gel-plus-column purification method is often unsuccessful for environmental samples with low biomass and/or large amounts of humic substances, resulting in low quality and low yield of DNA. Little improvement is achieved with modified incubation of the column at 80 °C for 10 min according to the manufacturer's recommendation. This motivates us to explore new strategies.

To reduce the amount of humic substances co-extracting with DNA, their solubilities at different pH values are considered. Humic substances are normally divided into three fractions (humic acids, fulvic acids, and humin) based on their solubilities as a function of pH. Humic acids are soluble in basic solutions but insoluble in acidic solutions; fulvic acids are soluble at any pH; humin is insoluble in water at any pH [24]. Therefore, use of acidic buffers should reduce dissolution of humic substances into buffers, and this leads us to use the PIPES buffer (with buffering range of pH 6.1−7.5 at 25°C [5]) to alter the Tris-HCl buffer (with buffering range of pH 7.9 to 9.0) for DNA extraction in the work. Our results suggest that the PIPES buffer (pH 6.5) has assuredly reduced the amount of humic substances co-extracting with the DNA, indicated by both color and absorbance values of crude DNA. Also, the PIPES buffer apparently improves the quality of the crude DNA measured by the ratios of $A_{260/280}$ and $A_{260/230}$. For example, the $A_{260/280}$ ratio of crude DNA extracted by the PIPES buffer is 1.787±0.007, and it is 1.363±0.030 for the Tris-HCl buffer (pH 8.0), indicating that a smaller amount of protein and polysaccharide materials presents in the crude DNA extracted by the PIPES buffer. However, the PIPES buffer results in lower DNA yields than the Tris-HCl buffer. This may be due to a reduction of HMW fraction of DNA as indicated by a lower DNA recovery with gel purification, and another explanation may be the less solubility of DNA in acidic PIPES buffers. The results indicate that the pH value of DNA extraction buffers may affect the quality and the yield of crude DNA.

Due to the fact that the crude DNA could not be directly used for whole community genome amplifications or PCR amplifications of 16S rRNA genes, the purification step for the crude DNA extracted by either the PIPES or the Tris-HCl buffer is necessary. Gel purification results in a high purity of DNA from all samples no matter whether the PIPES or Tris-HCl buffer is used. The Promega kit purification only produces high purity DNA from the crude DNA extracted by the PIPES buffer. However, such purified DNA cannot be stored for a long time, and it is observed that whole community genome amplifications and PCR amplifications are unsuccessful for some samples after one-month storage at −80 ºC (data not shown), suggesting an incomplete removal of contaminants. In contrast, all gel-purified DNA samples extracted by the Tris-HCl buffer can be successfully amplified by WCGA and PCR analysis of 16S rRNA genes and other functional genes (e.g., *dsr*, *nir*S and *nir*K, *amo*A) (data not shown) after four-month storage at −80 °C. Based on our results, either Qiagen or Omega kit is not recommended due to their low recoveries. These results suggest that gel purification is a robust method for the removal of not only humic substances, but also other contaminants which could degrade or modify DNA, especially when the amount of soil is a limited factor, and that the Promega kit purification is more suitable for PIPES-extracted DNA, especially when the amount of soil is a major concern and a long-term storage is not required.

In a number of DNA purification methods and protocols, gel separation is used [19, 25], which can separate DNA from contaminants such as humic substances and metals based on the differences of

molecular size and charge. Generally, gel separation is followed by gel digestion with GElase, or spin column filtration, resulting in a low yield and residual contamination of DNA with agarose gel and other components derived from buffers used. And the capacity is limited by enzyme and column used, thus they may not be used for a large scale DNA purification. Meanwhile, a DNA purification procedure combining agarose gel electrophoresis and spin columns is found to be superior to other methods with regard to removal of PCR inhibitors [19], but at the expense of low yields. The gel purification method used in this work can handle relatively large amount of DNA by using multiple, consecutive wells and cutting and pooling relatively large slices of gel with HMW DNA to a 15 mL tube, and then high purity of DNA can be obtained by extracting the gel using phenol, chloroform, and 2-butonal.

Two improved alternative methods are proposed from this work: the first with Tris-HCl extraction and gel purification, and the second with PIPES extraction and Promega kit purification. The first method is considered to be preferable for obtaining soil DNA with high quality despite relatively low yields, and it is specifically suitable for samples with enough amount biomass, for which a low DNA recovery is not a constraint. The second method is an alternative with relatively low quality but a high recovery, and it can be used for samples with low biomass or that partially degraded, which requires high DNA recovery. Our results show that most (>80%) of crude DNA is recovered by this method, because both sheared and HMW DNA fragments are recovered, suggesting that the high recovery may be at the expense of DNA purity. Therefore, these two methods can provide reliable DNA extraction and purification, which can be used for virtually any sample type, regardless of biomass, degradation or cleanliness level. Finally, it is noted that the DNA quality and final recovery also depend on many factors, such as sampling site, soil texture, soil transportation and storage, DNA extraction (e.g., HMW portion present in crude DNA) and purification, and storage duration. For example, high recoveries are achieved from 5 g of soil samples from the AC, SoyFace and PHACE sites, but not for the six soil samples from the OU warming site or soils from other sites.

5 Conclusions

1) All crude DNA extracted by two buffers contain HMW (≥ 23 kb) DNA. PIPES buffer can obtain high-quality crude DNA with low yield, while extraction with the Tris-HCl buffer can obtain high yield crude DNA but with low quality.

2) DNA yields are positively correlated with the soil amount with both Tris-HCl (r^2=0.94 and P=0.004) and PIPES (r^2=0.96 and P=0.002) buffers. In addition, the $A_{260/280}$ ratios of the crude DNA extracted by the Tris-HCl buffer correlate well with soil amount (r^2= 0.793 and P=0.04), but no significant correlation is observed when the PIPES buffer is used.

3) The Promega kit results in a higher recovery for Tris-HCl-extracted DNA ((82.23±1.72)%) than PIPES-extracted DNA ((76.0±4.51)%), but the $A_{260/280}$ and $A_{260/230}$ ratios of purified DNA are generally higher (1.72±0.03 and 1.91±0.01) for PIPES-extracted DNA than Tris-HCl-extracted DNA (1.66±0.01 and 1.45±0.02). Compared to the Promega kit and gel purification, poor recoveries are observed with the Qiagen ((13.31±1.75)% for the Tris-HCl buffer, and (8.09±0.26)% for the PIPES buffer), or Omega ((10.93±1.82)% for the Tris-HCl buffer, and (7.03±0.73)% for the PIPES buffer) kit.

4) Tris-HCl extraction and gel purification is robust and reliable, and the method with PIPES extraction and Promega kit purification may be chosen as an alternative when biomass is limited.

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